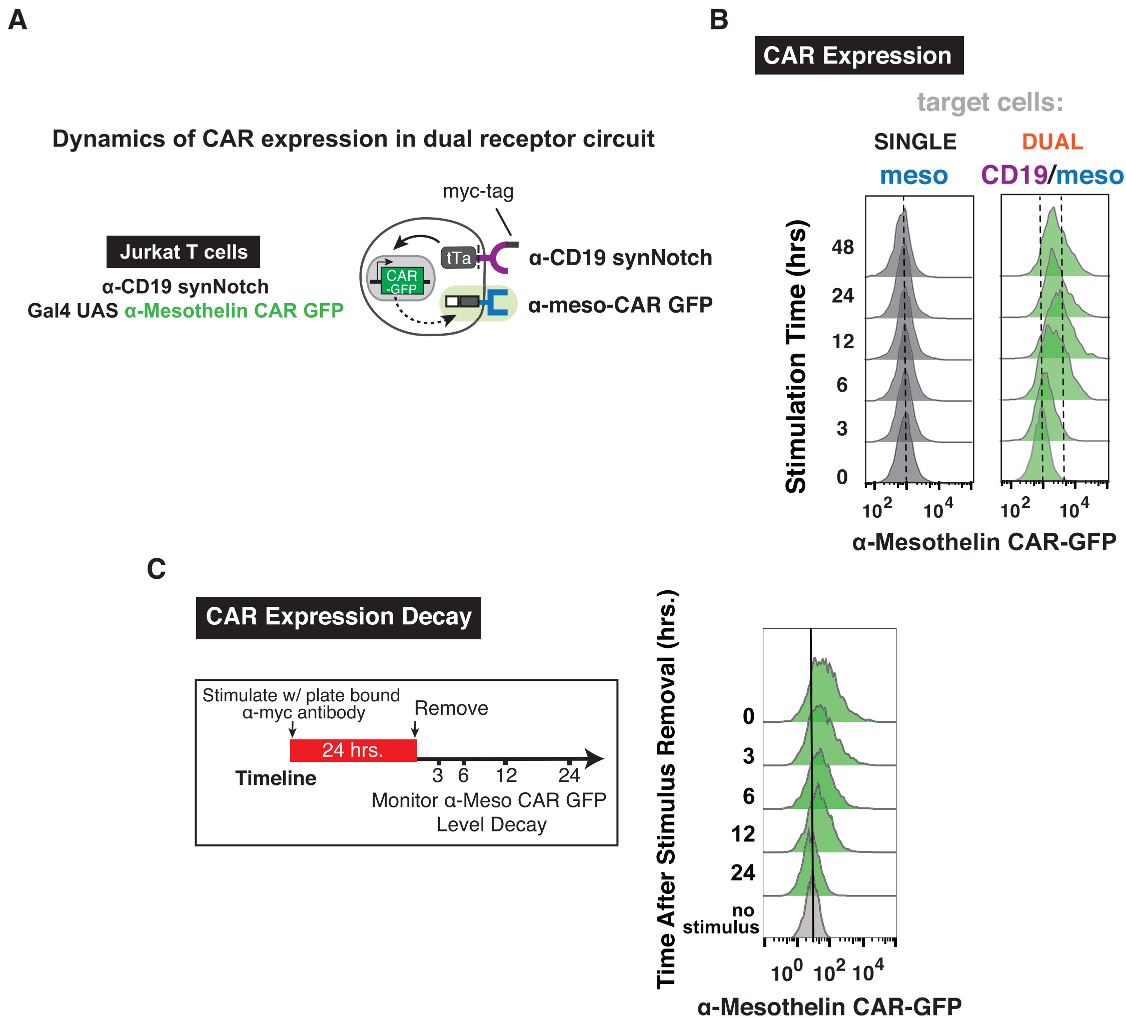

Figure S1. SynNotch-Gated CAR Expression—Combinatorial Antigen Requirement for Jurkat T Cell Activation, Related to Figure 2

(A) α-CD19 synNotch Jurkat T cells controlling expression of the α-mesothelin 4-1BBζ CAR fused to GFP (CD19 synNotch → Meso CAR) were incubated for 48 hr with Meso only or CD19/Meso K562s. Alternatively, the synNotch receptor could be activated by plate-bound anti-Myc antibody (the receptor has an extracellular Myc tag).

(B) Histograms of α-mesothelin CAR GFP expression in synNotch AND-gate Jurkat T cells co-cultured with single antigen (mesothelin only) or dual antigen (CD19/mesothelin) K562 tumor cells over 48 hr time course. CAR expression requires CD19 stimulation, and reaches steady-state with a $t_{1/2}$ of ~6 hr.

(C) SynNotch AND-gate Jurkat T cells were stimulated for 24 hr with plate-bound α-myc antibody (binds a Myc-tag on the extracellular domain of the synNotch receptor). After 24 hr, the cells were removed from the α-Myc stimulus and α-mesothelin CAR GFP expression decay was monitored for the subsequent 24 hr.

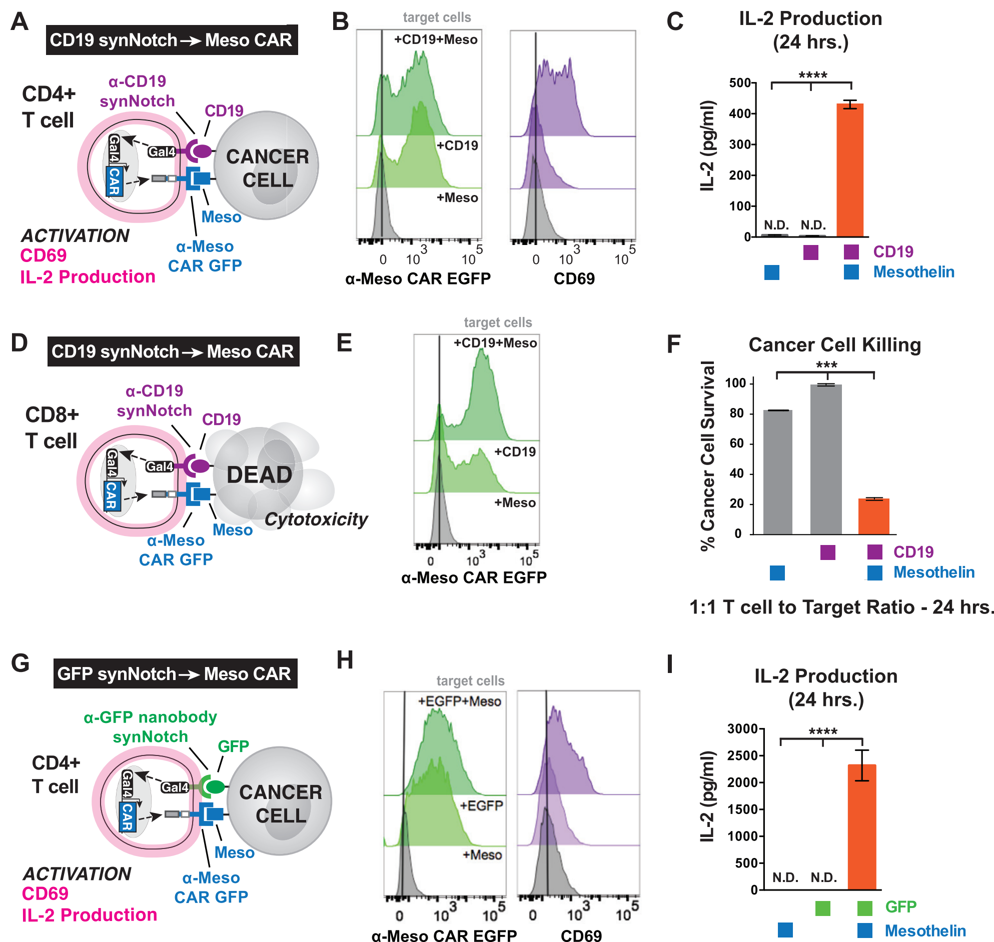

Figure S2. SynNotch-Gated CAR Expression in Human Primary T Cells—Combinatorial Antigen Control Over Therapeutic T Cell Activation and Tumor Killing, Related to Figure 3

This figure shows other examples of dual antigen circuits: *CD19 synNotch→ mesothelin CAR; GFP synNotch→ mesothelin CAR*.

(A) CD4+ primary T cells were engineered with the α-CD19 synNotch Gal4VP64 receptor and the corresponding response elements controlling α-mesothelin 4-1BBζ CAR GFP expression. The T cells were then co-cultured with mesothelin only, CD19 only, or CD19/mesothelin K562s for 24 hr and CD69 upregulation and IL-2 production were assayed.

(B) Histograms showing α-mesothelin CAR GFP levels and CD69 levels on CD4+ synNotch primary T cells cultured as described in (A). The α-mesothelin CAR was only expressed when CD19 was on the target K562s and the T cells only expressed the activation marker CD69 when both CD19 and mesothelin were on the target K562s (representative of 3 experiments).

(C) IL-2 levels from supernatant harvested from cultures described in panel (A). IL-2 was only produced when the T cells were exposed to target cells expressing both CD19 and mesothelin (n = 3, error bars are SEM, significance determined by Student's *t test* *** = $p \leq 0.001$).

(D) CD8+ primary human T cells were engineered as described in panel (A). For CD8+ T cells specific cytotoxicity of mesothelin only, CD19 only, or CD19/mesothelin target K562s was determined. The synNotch AND-gate CD8+ T cells should only kill dual positive K562s.

(E) Histograms showing α-mesothelin CAR EGFP levels on CD8+ synNotch primary T cells cultured as described in panel (A). The α-mesothelin CAR was only expressed when CD19 was on the target K562s (representative of 3 experiments).

(F) Quantification of replicate CD8+ synNotch AND-gate primary T cell cytotoxicity showing specific killing of target K562s with both CD19 and mesothelin expression (n = 3, error bars are SEM, *** = $p \leq 0.001$).

(G) CD4+ primary T cells were engineered with the α-GFP nanobody synNotch Gal4VP64 receptor and the corresponding response elements controlling α-mesothelin 4-1BBζ CAR GFP expression. The T cells were then co-cultured with mesothelin only, GFP only, or GFP/mesothelin K562s for 24 hr and CD69 upregulation and IL-2 production were assayed.

(H) Histograms showing α-mesothelin CAR GFP levels and CD69 levels on CD4+ synNotch primary T cells cultured as described in (G). The α-mesothelin CAR was only expressed when GFP was on the target K562s and the T cells only expressed the activation marker CD69 when both GFP and mesothelin were on the target K562s (representative of 3 experiments).

(I) IL-2 levels from supernatant harvested from cultures described in (G). IL-2 was only produced when the T cells were exposed to target cells expressing both GFP and mesothelin (n = 3, error bars are SEM, **** = $p \leq 0.0001$).

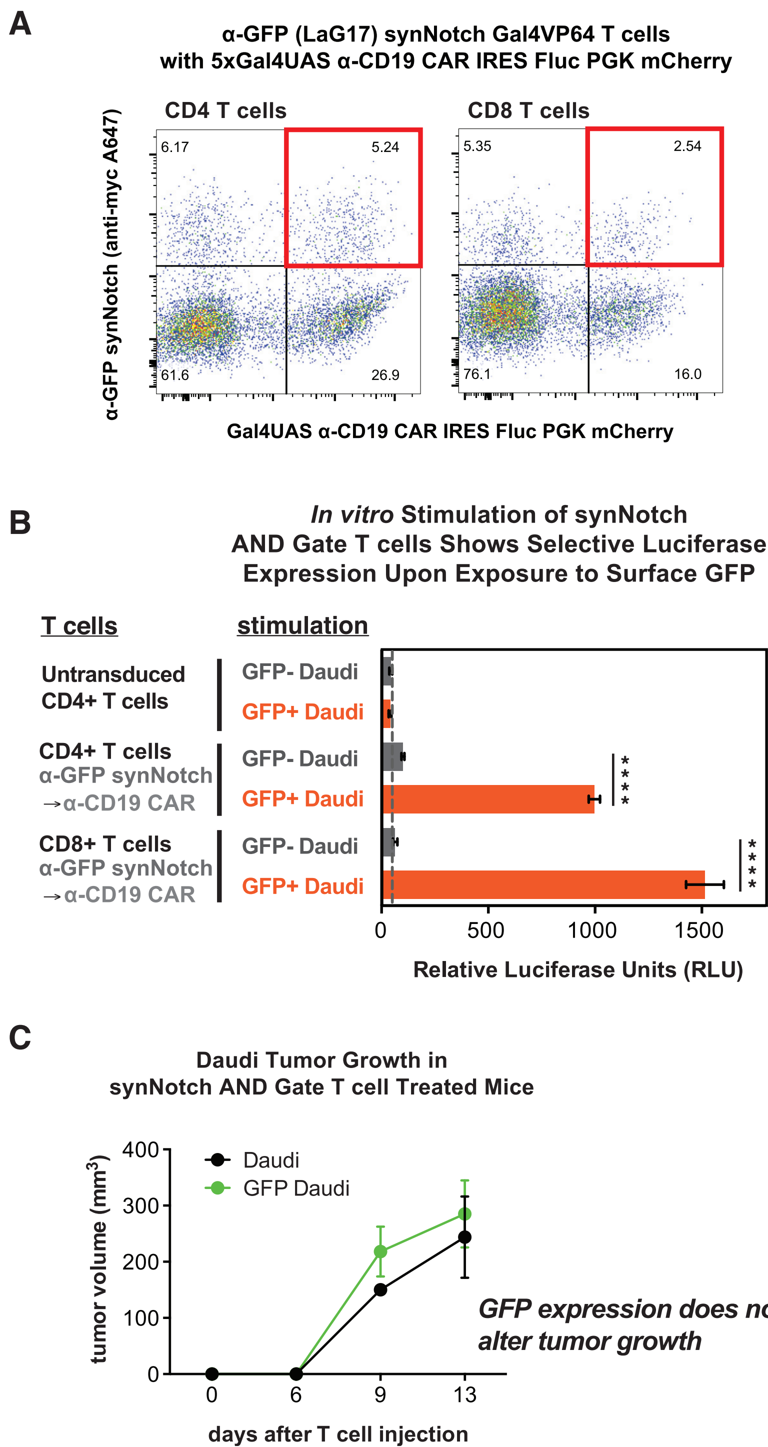

Figure S3. SynNotch Receptors Drive Tumor-Localized CAR Expression In Vivo, Related to Figure 4

(A) Representative dot plots showing expression of the α-GFP synNotch Gal4VP64 receptor and the corresponding response elements regulating α-CD19 4-1BBζ CAR IRES effluc in primary CD4+ and CD8+ T cells. The T cells outlined by the red box were sorted and used for in vivo and in vitro experiments.

(B) Bar graph showing luciferase activity in synNotch AND CD4+ and CD8+ T cells from (A) after exposure for 24 hr with GFP- or GFP+ Daudi cells. Luciferase was specifically expressed in response to GFP (n = 3, error bars are SEM, **** = $p \leq 0.0001$).

(C) Tumor growth curves are given for mice analyzed in Figure 4C.

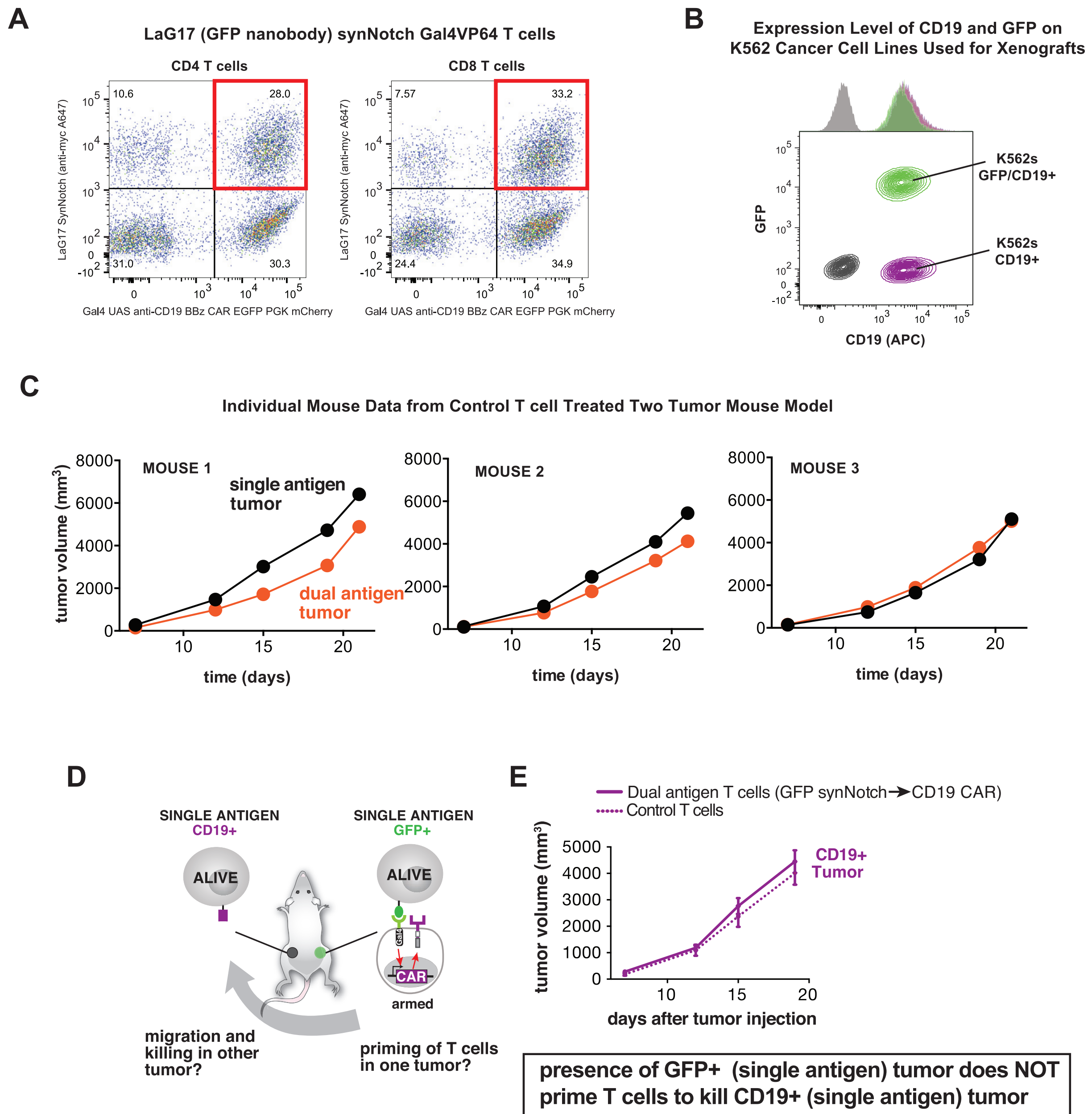

Figure S4. Selective Combinatorial Antigen Tumor Killing In Vivo by SynNotch-Gated CAR Expression, Related to Figure 5

(A) Representative dot plots showing the expression of the α-GFP synNotch Gal4VP64 receptor and the corresponding response elements regulating α-CD19 4-1BBζ CAR in primary human CD4+ and CD8+ T cells. T cells in the red-boxed quadrant were sorted and used for experiments in Figure 5.

(B) Flow cytometry plots showing the expression level of CD19 and GFP (green) on dual antigen K562s and CD19 on single antigen K562s (purple) utilized for in vitro and in *vivo* experiments.

(C) Tumor growth curves for individual mice with bilateral CD19 (left flank) and GFP and CD19 (right flank) tumors treated with control untransduced CD4+ and CD8+ T cells. The data underlie Figure 5B lower panel.

(D) Primary human CD4+ and CD8+ T cells were engineered with the α-GFP synNotch Gal4VP64 receptor and the corresponding response elements regulating α-CD19 4-1BBζ CAR expression and were injected i.v. into mice with a CD19 K562s on the left flank and a surface-GFP K562 tumor on the right flank to test if the T cells migrate from the GFP only 'priming tumor' and kill the off-target CD19 only tumor. Tumor size was monitored over 16 days after i.v. injection of engineered T cells or untransduced T cell controls.

(E) Graph showing CD19 tumor volumes for mice treated with synNotch AND-gate T cells (solid line) or untransduced control T cells (dotted line). The CD19 tumor is not targeted, suggesting there is no migration of primed T cells from the GFP only tumor (n = 5, error bars are SEM, no significant difference at any time points based on Student's t test, $p > 0.05$).